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Longevity of SLE-prone mice increased by dietary 2-mercaptoethanol via a mechanism imprinted within the first 28 days of life

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In the preceding report, moderately lived mice fed dietary 2-mercaptoethanol (2-Me) had their life extended, whereas long-lived mice were found to have the quality of life improved, but not extended, and did not develop high fat-diet obesity. In the present report, alteration of longevity of mice prone to develop spontaneous, systemic lupus erythematosus (SLE) by dietary 2-Me was determined. NZB, NZW, (NZW x NZB) F_1-hybrid, BXSB/MpJ, BXSB-*Yaa*$^+$/J, MRL/MpJ and MRL/MpJ-*Fas*^{lpr} mice received drinking water, without or with 2-Me at concentrations of 10^{-3} or 10^{-2} M. Therapeutic benefit was assessed by changes in longevity. The median survival of MRL/MpJ males was increased from 443 to 615 days and those of (NZW x NZB) F_1 and NZB males and females were increased approximately two-fold. The most unexpected finding was that longevity of F_1 males was significantly extended irrespective of whether dietary exposure to 2-Me was initiated at 28 days of age, at 50 days of age or initiated during gestation (and then terminated at weaning, 28 days of age). Survival of F_1-hybrids in which treatment was initiated in utero or at 28 days of age was not significantly different, whereas if initiation was delayed until 50 days of age, survival was >200 days shorter. Survival of male MRL/MpJ-*Fas*^{lpr} and BXSB/MpJ *(Yaa*$^-$), two strains with genetically controlled accelerated SLE, was not altered by 2-Me when started at 50 days. Various alternatives are discussed regarding potential long-lasting mechanisms imprinted early in life. Even though present day treatments of rodent SLE are generally aimed at controlling specific immunological events, with or without survival benefits or are procedures presently unsuitable for therapeutic use in humans, the findings presented herein seem worthy of clinical evaluation.

Introduction

We previously demonstrated[1-6] that optimization of murine immunological reactivity in tissue culture required a sulfhydryl compound; the most effective being 2-mercaptoethanol (2-Me). Since these reports, 2-Me was found beneficial for both growth/function of other cell-types in vitro including those of other species and when fed orally, impeded and/or reversed some in situ physiological changes associated with aging. More recently, thiol-containing compounds possessing oxidation-reduction potentials weaker than 2-Me were found to impart beneficial effects for human diseases (reviewed in ref. 7, in preceding report). Based on these effects, the research herein addressed the question: What consequences might dietary 2-Me impart on health and disease of mice other than those associated with aging?

Previous investigations indicate that (a) potential chronic and lethal toxicity[8] attributes of daily oral consumption of 2-Me did not negatively alter longevity,[7,9,10] most likely because the maximum average intake of 16 ugm per gm body weight spread over 24 hours was less than a LD_{50} bolus of 345 ugm per gm body weight,[8] (b) in situ age-related functions were prevented/reversed,[9-12] (c) appearance of cancer was slowed,[10,11] (d) survival of long-lived mice[10] and moderately lived mice was extended,[7] (e) high-fat diet obesity was curtailed,[7] and (f) a high quality of life was maintained by preventing recumbent, emaciation and cachectic health aspects associated with aging.[7] The present report is an extension of in situ investigations on 2-Me; namely, to assess alteration of longevity of mice that are prone to develop spontaneous, autoimmune-like, systemic lupus erythematosus (SLE).

Results

Because of potential toxic effects of 2-Me and since it is generally accepted that death of SLE-prone mice is a consequence of autoimmune-incited renal failure, the only SLE parameter monitored was survival. As a reference for presentation, median life spans found for strains treated with 2-Me are shown in **Table 1**.

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Table 1. Median life span of 2-Me-treated/non-treated strains of mice

Strain/sex	SLE nephritis[a]	Median survival day—treated with		
		nothing	10^{-3} M 2 Me	10^{-2} M 2 Me
(NZW x NZB) F_1				
male	++	349 (15)[d]	664 (9)[d]	750 (11)[d]
female	++	362 (9)	522 (8)	619 (11)
NZB				
male	++	350 (10)	664 (9)	655 (18)
female	++	331 (7)	602 (17)	nd
NZW				
male	-	916 (15)	760 (9)	870 (8)
female	-	756 (5)	527 (6)	840 (10)
MRL/MpJ				
male	+	443 (5)	nd	615 (5)
MRL-*Fas*^{lpr}				
male	+++	173 (5)	nd	209 (5)
BXSB-Yaa^{+}				
male	-	650 (5)	nd	954 (5)
BXSB/MpJ				
male	+++	258 (5)	nd	233 (5)
C57BL/10[c]				
male	-	957 (12)	981 (12)	nd
B10.A (4R)[c]				
male	-	895 (22)	933 (22)	nd
A/J[c]				
male	-	434 (7)	576 (9)	nd

[a]severity of nephritis as defined by numerous reports in the literature.
[b]nd = not done. ref. 7. [d]#animals in ( )—only changes >125 days were significant.

The results shown in **Figure 1** are those found for (NZW x NZB) F_1-hybrids started on treatment at the time they were weaned (28 days of age). As shown in **Figure 1A**, mean survival of females (parents **were not** on 2-Me) was significantly increased ($p = 0.019$ and $p = 0.006$) by both 10^{-3} and 10^{-2} M 2-Me (median time also increased, **Table 1**). Mean survival at the two 2-Me concentrations was not significantly different. Likewise, mean survival of males (parents were not on 2-Me) was also significantly increased by 10^{-3} and 10^{-2} M 2-Me ($p = 0.0006$ and 0.0002). Again, median survival increased and survival at the two 2-Me concentrations was not significantly different.

Survival shown in **Figure 1C** is that of offspring of parents/grandparents that were on 10^{-3} M 2-Me water their entire lives. The F_1-hybrids for this experiment were potentially exposed to 2-Me in utero, during lactation, and to any water consumed prior to weaning at 28 days. At weaning, offspring from these treated parents were continued on 10^{-3} M ($n = 10$) or switched to either untreated water ($n = 6$) or to 10^{-2} M water ($n = 7$). Surprisingly, mean survival (and medians) of offspring that were switched to untreated water (median 693 days) was statistically indistinguishable ($p = 0.29$ and 0.35) from that of those switched to 10^{-2} M or those continued on 10^{-3} M (medians of 704 and 723).

Natural demise of untreated (NZB x NZW) F_1-hybrid and NZB mice is generally accepted to be due to renal failure. Since survival of untreated NZB and the reverse F_1-cross used herein is similar to those published, it is assumed that their deaths were also due to renal failure. Further, no visible tumors were noted in untreated animals. In contrast, two of 17 F_1-hybrid animals treated with 10^{-3} M 2-Me starting at 28 days of age and two of 22 treated with 10^{-2} M died with cancer (shown by "+" in the **Figures**); all but one at >500 days.

Longevity of treated/non-treated, F_1 parental strains and two other unrelated, autoimmune-SLE-prone strains was determined. As shown in **Figure 2A and C**, mean survivals of NZB females ($p = 0.010$) and males ($p < 0.0009$) were significantly increased by 2-Me; median survivals were also increased (**Table 1**). No tumors were observed for treated or non-treated NZB animals.

In contrast to NZB and F_1-hybrid animals, NZW females (**Fig. 2B**) and males (**Fig. 2D**) treated with 10^{-3} M had shorter mean survival times (significant for males, $p = 0.021$, but not for females, $p = 0.085$) than non-treated controls (median survivals were also shorter, **Table 1**), whereas mean and median survival of those treated with 10^{-2} M were similar to those of non-treated animals. Survival of NZW females and males treated with 10^{-2} M was extended compared to that of those treated with 10^{-3} M (however only females were significantly extended). A low incidence of cancer was found for this strain, irrespective of treatment—controls (1 of 20), 10^{-3} M (3 of 15) and 10^{-2} M (1 of 18).

As shown in **Table 2**, the amount of a 6% fat diet consumed by control and 10^{-3} M 2-Me-treated C57BL/10 males was similar. However, these mice consumed considerably more 2-Me water relative to normal water. Similar results were found with (NZW x NZB) F_1 mice at a similar age; mice on 2-Me consumed the same amount of food (same 6% fat diet fed B10) and more 2-Me water than control mice. The average ugm of 2-Me consumed/day/gm body weight by F_1-hybrids was similar to that consumed by B10 mice.

Figures 3 and 4 show the survival of males of the autoimmune accelerated SLE-prone strains, MRL/MpJ-*Fas*^{lpr}/J and BXSB/MpJ, and their congeneic, control strains, MRL/MpJ (which develops a late, mild-form of SLE[13]) and BXSB-*Yaa*$^{+}$/J (which does not develop SLE), started on 10^{-2} M 2-Me at 50 days of age (the day they arrived from Jackson Laboratory). As shown in **Figure 3**, 2-Me had no significant beneficial or detrimental effect on survival of mice with the mutant *lpr* gene. Even though median survival of the congeneic control, MRL/MpJ, was increased by 2-Me, the mean survival was not significantly different ($p = 0.11$); primarily due to the survival of a single control animal and to the small number of animals tested.

Figure 4 shows survival changes that occurred in male BXSB and its congeneic partner treated or not treated with 10^{-2} M 2-Me (treatment started at 50 days). The accelerated demise of BXSB mice, carrying the *Yaa* gene on the Y chromosome was not altered, whereas survival of the congeneic partner was dramatically increased ($p = 0.006$).

An explanation as to why 2-Me was ineffective for the two accelerated models of SLE is that treatment started at 50 days of age is just too late relative to initiation of disease. This was tested by determining survival of F_1-hybrid males (for which 2-Me was effective) started on treatment at different ages. Shown in **Figure 5**, starting in utero, at 28 days or 50 days of age resulted in significant increases in survival (p of 0.007 to <0.0001) vs. that of controls. However, 2-Me treatment instituted early increased longevity more, with no significant difference between in utero and 28 days of age.

Discussion

Systemic lupus erythematosus in mice and humans[14,15] is a chronic systemic autoimmune disease that is influenced by undefined environmental factors and is under control of a multitude of genetic loci that contribute to susceptibility by epistatic interactions.[16-19] Moreover, even though SLE disease may involve multiple organ systems, glomerulonephritis, which can lead to fatal renal failure, is considered the most serious. Presently, NZB, (NZB x NZW) F_1-hybrid, MRL/MpJ-*Fas*^{lpr} and BXSB (males only) strains of mice have been the most extensively studied as models for human SLE; other congeneic, transgenic, F_1-hybrids and knockout models are increasingly being studied. In the present report, only these four strains, plus the other F_1 parental strain, NZW and where appropriate congeneic strains were studied.

Because of potential toxicity of 2-Me and because certain SLE phenotypic parameters are not always associated with survival,[20-22] survival, presumed to be a consequence of renal failure, was the only phenotypic change chosen to be monitored in the present investigation. It was reasoned that if any significant increase in longevity occurred, additional research could be undertaken to define which other phenotypes might be altered by 2-Me and their role in longevity changes. Findings of significance were: (a) longevity was increased when 2-Me treatment (i) was started at 28 days of age for NZB and for (NZW x NZB) F_1-hybrids, (ii) was started at 50 days of age for (NZW x NZB) F_1-hybrids and for MRL/MpJ, (the latter strain develops SLE more slowly[13] than the

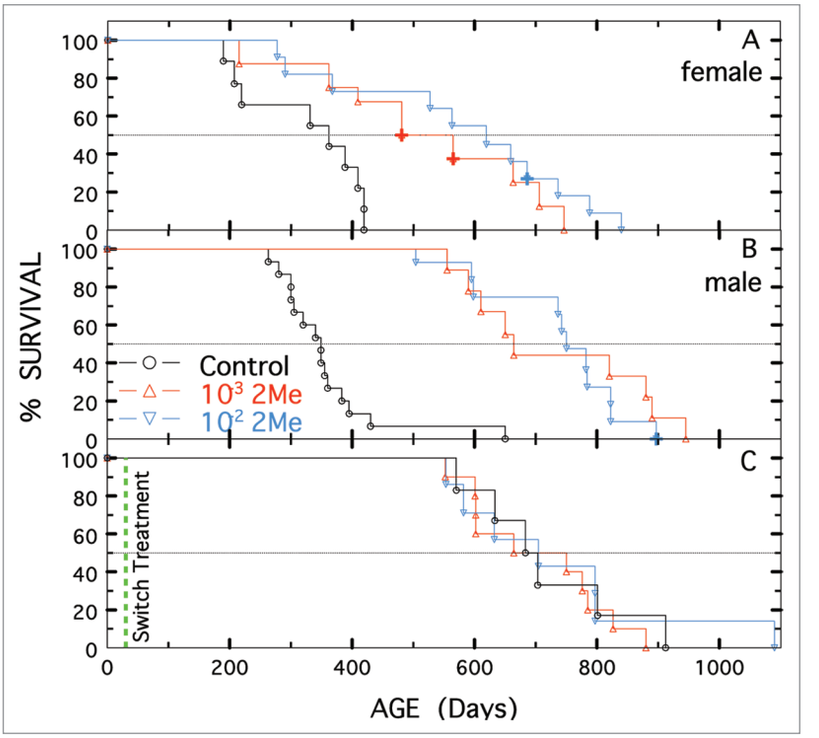

Figure 1. Longevity of (NZW x NZB) F_1-hybrid male and female mice exposed to normal, 10^{-3} M or 10^{-2} M 2-Me drinking water. (A) Females. Treatment started at 28 days of age. Nontreated water shown in black (n = 9), 10^{-3} M 2-Me water in red (n = 8) and 10^{-2} M 2-Me water in blue (n = 11). (B) Males. Treatment started at 28 days of age. Control (n = 15), 10^{-3} M 2-Me (n = 9), 10^{-2} M 2-Me (n = 11). (C) Males. Grandparents and parents on 10^{-3} M 2-Me their entire lives. At 28 days of age, water of offspring of these parents/grandpaarents was switched to: nontreated water (n = 6), 10^{-2} M 2-Me (n = 7), or continued on 10^{-3} M 2-Me (n = 10) for the remainder of their lives. Animals with solid tumors or ascites are designated by +.

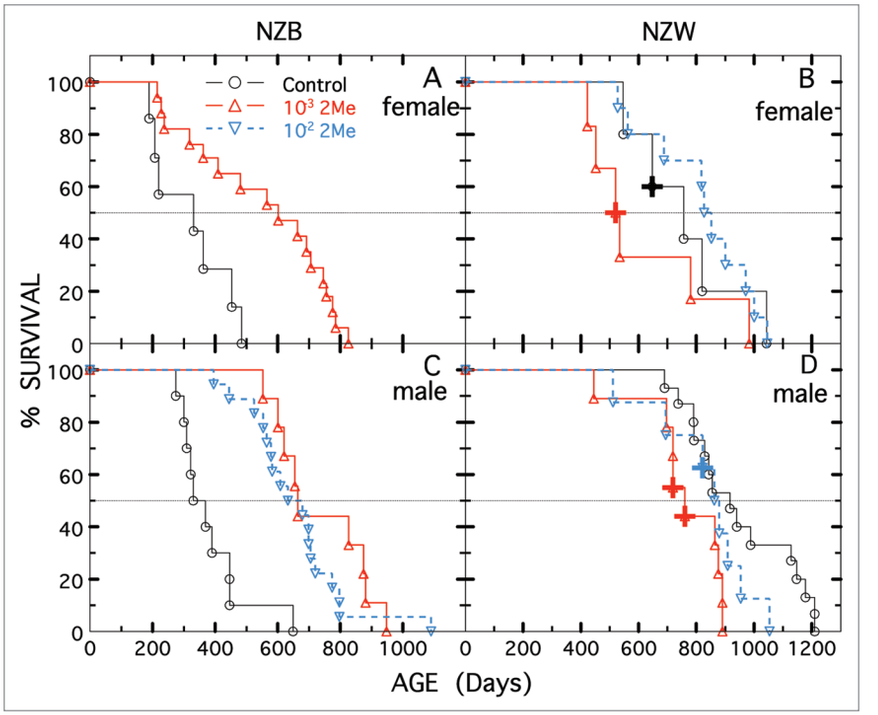

Figure 2. Longevity of NZB and NZW mice not treated or treated with 10^{-3} or 10^{-2} M 2-Me. (A and B) Females. (C and D) Males. Treatment started at 28 days of age for control shown in black, 10^{-3} M 2-Me in red and 10^{-2} M 2-Me in blue. Animals with solid tumors or ascites are designated by +.

Table 2. Body weight and average daily intake of food, water and 2-Me by male (NZW x NZB) F_1 and C57BL10 mice at 210 days of age

Ave/mouse/day	(NZW x NZB) F_1[a]		C57BL10[a]	
	minus 2 Me	$+10^{-3}$	minus 2 Me	$+10^{-3}$
Water (ml)	7.7	8.3	3.7	6.2
Food (gms)	5.5	5.6	4.1	3.8
Body wt (gms)	43.2	42.2	35.8	32.2
2-Me (ugm)	0	650	0	484
(ugm/gm bw)		15.3		15.0

[a]group of 6 animals/cage/variable.

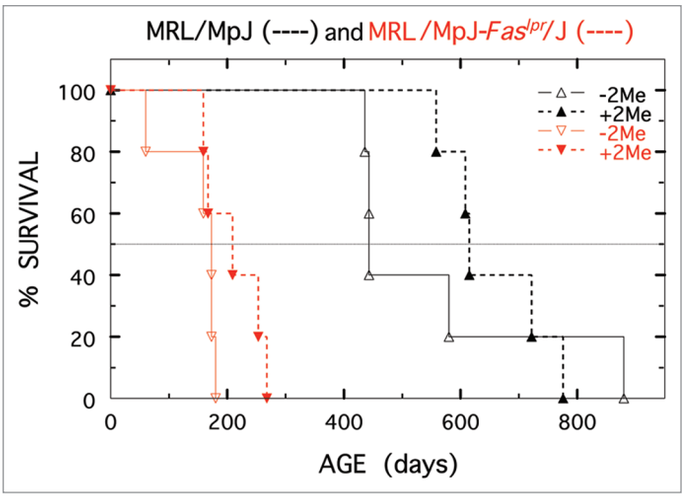

Figure 3. Longevity of MRL/MpJ (shown in black) and MRL/MpJ-*Fas*^{lpr}/J (shown in red) males not treated (open triangles) or treated with 10^{-2} M 2-Me starting at 50 days (closed triangles). Five mice per treatment.

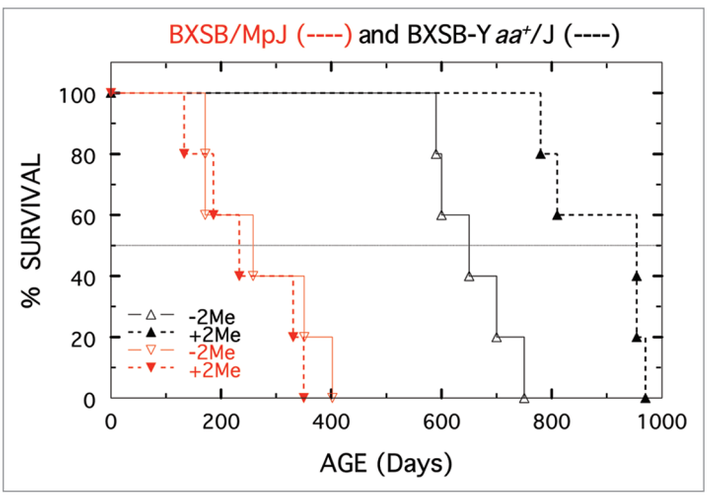

Figure 4. Longevity of BXSB-*Yaa*$^+$/J (shown in black) and BXSB/MpJ (shown in red) males not treated (open triangles) or treated with 10^{-2} M 2-Me starting at 50 days (closed triangles). Five mice per treatment.

other two), but was ineffective (started at 50 days) at extending survival of animals with genetically-controlled, accelerated SLE; (b) longevity of F_1-hybrids was inversely associated with the age at which treatment was started—treatment started in utero or at 28 days of age resulted in similar increases in longevity, whereas when started at 50 days, survival was >200 days less; (c) 2-Me imprinted a long-term survival benefit for F_1-hybrid males potentially exposed to 2-Me only in utero, via lactation (similar to the maternally transmitted autoantibodies that curtailed diabetes in NOD progeny[23]), or days prior to weaning; (d) median survival of NZW mice, the F_1-parental strain that does not develop nephritic SLE even though it carries SLE susceptibility genes, was shortened by 10^{-3} M 2-Me—for females from 756 to 527 days and for males from 916 to 760 days. In contrast, treatment with 10^{-2} M 2-Me did not significantly shorten or extend survival of NZW of either sex.

These findings raise a number of intriguing questions regarding 2-Me's mechanism of action. First, was the increased longevity associated with 2-Me treatment a consequence of (a) preventing glomerulonephritis, (b) merely slowing progression of disease or (c) alteration of some other event that was lethal? Second, by what mechanism(s) did 2-Me extend survival of strains with relatively moderate or slow developing SLE but not those possessing the accelerating, *lpr* or *Yaa* genes? Third, what mechanism was imprinted within 28 days of birth by 2-Me that persisted for a lifetime? Fourth, how did 2-Me shorten the life span of NZW at 10^{-3} M but not at 10^{-2} M.

Based on the magnitude of change in longevity, it is presumed that it was in some manner a manifestation of extensively reduced nephritis (perhaps completely). Assessment of genes that are common to the various strains was not informative to explain the selectively extended survival. Possibilities being considered are; (a) an effective 2-Me treatment must be initiated prior to triggering the disease process (for strains raised in house, this was at least 28 days or earlier) and for the two accelerated SLE models, treatment started at 50 days (when they arrived from Jackson Labs) is simply too late, (b) products/factors/pathways controlled by the two accelerating genes, *lpr* and *Yaa*, resist/bypass 2-Me imparted survival mechanisms of the other strains (is death due to failure of a different organ system as suggested for Crry-Ig-treated *lpr* animals[24] or to dysregulated lymphoproliferation) and (c) others yet to be defined. Support for the first postulate is the 2-Me extended survival of the slower-development of SLE in the *lpr*-parental strain, MRL/MpJ (treatment started at 50 days), as well as the much poorer extension of F_1-hybrids started at 50 days compared to that of those started in utero or at 28 days of age. Further analysis with other SLE-prone *lpr*-strains started on treatment at different ages and monitoring various SLE phenotypic markers should be informative.

The third finding, perhaps of greater significance, is that longevity of treated F_1-hybrid males (median survival of 349 for nontreated) was independent of whether treatment was started at 28 days of age (medians of 664/750 days at 10^{-3}/10^{-2} M) or started in utero and stopped at 28 days of age (median of 693 days), or continued post weaning (medians of 723/704

days at $10^{-3}/10^{-2}$ M). Interesting mechanisms to consider for this prenatal/early life, long-lasting imprinting, which may also underlie the increased longevity of animals started on treatment at weaning are, 2-Me: (a) alters parental (with emphasis on the maternal parent) SLE-inducing environmental factor/etiologic agent(s) or commensal intestinal flora[25,26] resulting in flora (or products) of offspring that mimics that of the parents; (b) interferes with pathogen recognition (initiation phase) via T or B cell receptors, and/or innate receptors, such as TLR7 and TLR9,[27-31] (c) alters a specific innate or adoptive pathway component post etiologic recognition and (d) replaces[32] or programs[33-35] antigen presenting dendritic cells[36] so that an homeostatic change (dysfunctional[33] or deficient[37-40] correction) occurs in regulatory cells to favor tolerance relative to effector autoimmune T cells as a consequence of IL-2 deprivation.[39] Any of these postulates, especially the one in which regulatory cells are induced to play a toleragenic role, are plausible. Indeed, any could easily be achieved by reduction of essential disulfide bonds (or curtailing formation of new ones) by 2-Me. For example, alterations that create molecular and/or cellular modifications, such as cleavage of autoantigens by presumed cysteinic proteases[41-43] during the process of apoptosis, could prevent the presentation of alternative epitopes[44] to which tolerance is absent. Such changes could alter SLE-initiation, or for that matter, any other autoimmune disease that has an environmental link. Experiments with appropriate strains of mice on defined diets and with specific flora should answer whether this has any relevance for extension of the life span of SLE-prone mice by 2-Me.

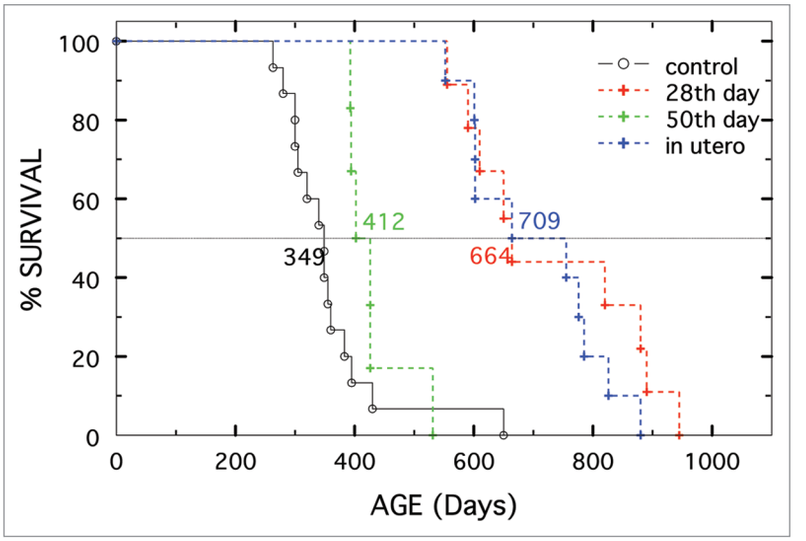

Figure 5. Survival of (NZW x NZB) F_1 males started on 10^{-3} M 2-Me at different ages. Not treated shown in black (n = 15). Started in utero shown in blue, (n = 10). Started at 28 days of age shown in red (n = 9) and started at 50 days shown in green (n = 6).

And finally, by what means was survival of NZW mice shortened by 10^{-3} M but not by 10^{-2} M 2-Me drinking water? A similar shortening was found for leukemic-prone, AKR/Cum, mice treated with 5 x 10^{-4} M, but not those treated with 10^{-3} or 10^{-2} M (Click RE, unpublished). An interesting possibility is that alteration of crucial protein SH/disulfide moieties could lead to activation/inactivation of endogenous viruses and/or their products.[45]

No tumors were observed in long-lived (treated) or short-lived (untreated) NZB animals; the latter most likely because of its short life span. In contrast, 2-Me treatment did not alter the low incidence of tumors in long-lived NZW and F_1-hybrids; although fewer animals with tumors were observed in those treated with 10^{-2} M than with 10^{-3} M 2-Me. These results support the findings of others in which the development of tumors was slowed by 2-Me exposure.[10,11] How 2-Me effectively prevented cancer in other cancer-prevalent strains is the focus of a future report.

Although numerous gene manipulations or immunotherapy regimens, deemed by many as potential targets, alter development of SLE,[46-48] limited progress has been achieved towards controlling autoimmunity in general.[49] Indeed, at present, there are two clinically applicable protocols that result in long-term survival ("cures") of SLE mice; allogeneic bone marrow transplantation[50,51] and a diet of fish oil containing a high concentration of the n-3 fatty acid, docosahexaenoic acid.[52] Other treatments, such as retinoic acid supplementation,[53] alteration of IFNγ, IFNγ receptors[54] and/or FcγRIIB,[55] addition of a class II, I-E molecule to strains that lack this gene,[56] B cell depletion[57,58] are designed as interventions aimed at controlling specific immune aspects of the disease, with or without long-term survival benefits or are protocols presently unsuitable for therapeutic use in humans. Thus, there remains a need for clinically applicable protocols that prevent development of SLE as well as cure-established SLE. The findings presented herein add a simple modality, presumably preventive, that intervenes with an early event triggered by an etiologic SLE-inducing-agent(s), worthy of clinical evaluation. This is especially intriguing in that a comparable genetic defect in *fas/apo-1* of *lpr* mice (in which 2-Me was ineffective) is absent in humans with SLE.[59]

Conclusions

As therapeutic, nontoxic, in situ levels of 2-Me become better defined, it is anticipated that a more thorough understanding of the mechanisms of its alteration of fat-induced obesity,[7] maintenance of end-stage high-quality of life,[7] increase in longevity of non-SLE[7,10] and SLE-prone strains and slowing the development of cancer[10,11] will be extended with the multitude of genetically defined strains of mice and available specific blocking and activating factors. It will be of interest to establish whether 2-Me (a) acts via a systemic metabolic pathway, such as maintenance of immune homeostasis systems, (b) alters environmental factor(s) that are associated (causative) with disease, (c) scavengers free radicals associated with autooxidation and aging, (d) acts by some combination of these or (e) by other presently undefined, alternatives. In addition, whether 2-Me can effectively treat established

SLE, as well as prevent/alter other autoimmune diseases, such as diabetes, Crohn's and arthritis is of considerable interest.

Materials and Methods

Mice and their husbandry. Inbred strains of NZB, NZW, BXSB/MpJ, BXSB-*Yaa*$^+$/J, MRL/MpJ and MRL/MpJ-*Fas*^{lpr} were purchased from Jackson Laboratory, Bar Harbor, ME. From these strains, experimental male and female NZB, NZW and (NZW x NZB) F_1-hybrids (note this cross is the reverse of that routinely used by others) were derived from our breeding colony. All mice were housed in standard Plexiglas ventilated conventional boxes (4–5 animals/box) within a facility that maintained a 12 hour light/12 hour dark cycle. All animals in the study succumbed from natural causes and had free access to food (Harlan Teklad mouse/rat 6% fat diet) and to autoclaved distilled/deionized water with or without added 2-Me. All experiments were performed in accordance with institutional animal research guidelines approved by the VA IACUC.

Water. Sterile, deionized/distilled water for drinking was supplied in glass bottles, which were changed, cleaned and autoclaved twice a week. For animals treated, 2-Me was added to each bottle to obtain a final concentration of 10^{-3} or 10^{-2} M on the day the bottles were changed. Consumption of water was measured over a 3.5-day interval, corrected for the number of animals per cage and expressed as average ml/day/mouse.

Feed. The amount of feed consumed over a seven day period was measured, corrected for the number of animals per cage and expressed as average gms/day/mouse.

Statistics. The Mann-Whitney U test was used to assess differences in mean survival. For all comparisons, p values less than 0.05 were considered to be statistically significant.

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